RYEGRASS STAGGERS IN THE NETHERLANDS

Ryegrass staggers is a syndrome caused by Lolitrem B, a mycotoxin produced by the endophyte Neotyphodium lolli in horses and ruminants. Clinical signs of the disease in horses are tremors, head shaking, incoordination, staggering, swaying gait, unstable standing and fasciculations of the neck and limbs. The aim of the study was to gain a better insight of the risk of Lolitrem B exposure and adverse effects in horses, with special emphasis on the situation in Netherlands. To this end the literature was reviewed and data from the analysis of hay samples were compiled. Results show that the risk of a lolitrem B intoxication is low in The Netherlands, and confined to individual cases in late autumn. However, Lolitrem intoxications remains an important differential diagnosis in daily equine practice having in contrast to other neurological diseases a very good prognosis for entire recovery of the affected animal.

Content

Abstract

Ryegrass staggers is a syndrome caused by Lolitrem B, a mycotoxin produced by the endophyte *Neotyphodium lolli* in horses and ruminants. Clinical signs of the disease in horses are tremors, head shaking, incoordination, staggering, swaying gait, unstable standing and fasciculations of the neck and limbs. The aim of the study was to gain a better insight of the risk of Lolitrem B exposure and adverse effects in horses, with special emphasis on the situation in Netherlands. To this end the literature was reviewed and data from the analysis of hay samples were compiled. Results show that the risk of a lolitrem B intoxication is low in The Netherlands, and confined to individual cases in late autumn. However, Lolitrem intoxications remains an important differential diagnosis in daily equine practice having in contrast to other neurological diseases a very good prognosis for entire recovery of the affected animal.

Introduction

In equine medicine, owners, trainers and veterinarians are confronted with a broad array of neurological and muscle diseases that seriously affect animal health and wellbeing (Goehring et al., 2005). The causative agents and pathophysiology of these diseases vary from viral infections, to the ingestion of toxic plants or mycotoxins, to metabolic diseases with neurological signs (for review see Aleman, 2001; Rech & Barros, 2015). Many of these clinical cases have a poor prognosis and the possibilities for therapeutic intervention remain limited. In the differential diagnosis of clinical symptoms such as muscle tremors, apparent lameness, excessive sweating and ataxia, the syndrome of ryegrass staggers can appear. This disease, caused by ingestion of freshly harvested endophyte-infected ryegrass hay or flowering pasture grass at the end of the grazing season, may cause an acute intoxication in horses, attributed to the mycotoxin Lolitrem B. The symptoms range from hardly recognisable to severe ataxia and inability to move (Johnstone et al., 2012).

In this survey the risk of Lolitrem B intoxication in horses will be studied. The aim of the study is to gain a better insight of the risk of Lolitrem B exposure and adverse effects in horses, focussing on the situation in the Netherlands. Lolitem B is produced by the endophyt *Neotyphodium lolii* that is common in perennial ryegrass. Since Lolium perenne is one of the most commonly found grass species in the Netherlands, Lolitrem intoxications have been reported in The Netherlands. Here we present a literature review on the different aspects of lolitrem B synthesis and occurrence, the adverse effects that may occur in horses following the ingestion of Lolitrem-contaminated hay. In addition, we investigated in one season a number of hay samples and compared these with results obtained in the same lab over a period of 10 years.

Endophytic fungi

The large kingdom of *fungi imperfecti* (Deuteromycetes) are often divided in five groups, the epiphytic (common visible molds), the endophytic (living invisible in the plant), the mycorrhizal (important soil-born fungi living around the roots of a plant and being essential in

nutrient transport to the plants), the pathogenic (living in and on tissues, like the epidermis or the lungs and other body cavities) and the saprotrophic fungi, the common mushroom species (also called *fungi perfecti*) that can be edible or produce distinct classes of fungal toxins such as amantadins, which are clearly distinguished from the large group of mycotoxins.

Endophytes of the genus Epicloë comprise a distinct, and specific group as they live in a true symbiosis with living plants. Endophytic fungi are different from all other fungal species as they reside in the plant tissue and are therefore are not visible. (Porras-Alfaro & Bayman, 2011; Schardl, 2001) Endophytes have a mutualistic relation with the plant that is infected. Endophytes are able to produce a variety of secondary metabolites, that are beneficial for the plant. A wide range of insect-repellent, antifungal, anthelmintic and antibacterial substances have been isolated from endophyte cultures, which can protect the infected plant against infections with pathogenic microorganisms, helminths and insects. Actually in some countries it is essential for certain grasses to be protected by endophytes, as for example ryegrasses in New Zealand. Ryegrass in New Zealand would not survive the Argentine stem weevil and the African black beetle invasions if not protected by f endophyte-derived peramines and lolines. The production of secondary metabolites by endophytes is influenced by the physiological state of the host-plant (growth phase, flowering, drying), in contrast to epiphytic fungi, for which nutrient availability, temperature and humidity are the main drivers for invasion and sporulation. The specific characteristic of an endophyte and epiphytic fungi are summarized in Table 1.

Table 1: *Overview of the main differences between endophytic and other (phytopathogenic) fungi*

Endophytic fungi	Epiphytic fungi
Entire lifecycle in internal tissue of the host-plant	No full lifecycle in plant tissue, wide range of substrates.
Generally not pathogenic for the host-plant	Both pathogenic and non-pathogenic for the host-plant
Periodic growth depending on the host-plant life cycle	Growth independent of the host-plant and on a variety of substrates
Mycotoxin production mainly influenced by host-plant	Mycotoxin production mainly influenced by environmental factors

Another beneficial effect of the endophytes for the plants is the beneficial effect of *Neotyphodium lolii* on the production of plant biomass and a higher tolerance for drought. (Bacon, 1995; Gilbert et al., 2012; Porras-Alfaro & Bayman, 2011)

Mycotoxins and mycotoxicoses in horses

In the 1930's a yet unknown disease seen in horses was studied in the Ukraine. The researchers got their first clue when they noticed that only the horses of the local farms where sick and not the horses of the army. So they understood that it had to do with the forage, since the horses of the army did not get the same forage as the horses of the locals. The researchers

noticed a black coating on the straw, which was fed to the horses of the local farmers. The black coating was formed by a fungus and it is very likely that secondary metabolites of this fungus did cause the disease, even though the researchers in the 1930's did not have the means to prove the presence of such substances. Only in 1968, when a yet unknown disease in turkeys was associated with imported peanut meal, chemical analysis by thin-layer-chromatography confirmed for the time, the link between a disease in animals and the presence of fungal metabolites (mycotoxins) in the feed material.

Mycotoxins are secondary metabolites synthesized by different classes of fungi and they are considered to convey benefits to the fungus in a competitive environment (see for example the antibacterial effects of many fungal-derived antibiotics and mycotoxins). Mycotoxins are chemically stable, and they resist common food and feed processing methods like cooking and heating (Bullerman & Bianchini, 2007) and acidification. Therefore it is virtually impossible to remove mycotoxins that are present in forage.

Probably the most well-known and well-described mycotoxicosis in horses is the so-called Equine Leuko-Encephalo-Malacia (ELEM) following the ingestion of yellow corn (maize) contaminated with Fumonsins, of which Fumonisisn B1 is considered to be the most prevalent and most toxic form. Fumonisin B1 (together with other related fumonisin-derivatives) is produced predominantly by *Fusarium virticilloidis*, which only infests maize plants and the highest toxin concentrations are found in the cob and the (ripened) yellow kernels. ELEM is frequently seen in the United States and has high mortality rates in horses (Domijan, 2012; Liesener et al., 2010). Fumonisin B1 is found also frequently in corn cobs and kernels in Europe, but here the concentrations are so low, that the risk for animals seems to be negligible. However, probably as part of the global climate changes, recently incidental mild intoxications have been reported in France and Spain.

Other mycotoxins that are of importance in the diet of horses include Aspergillus toxins, such as Aflatoxin B, different other Fusarium toxins, including trichothecenes and Zearalenone, Penicillium toxins and ergot alkaloids. (for review see Caloni & Cortinovis, 2011; Caloni & Cortinovis, 2010; Domijan, 2012; Marin et al., 2013; Riet-Correa et al., 2013)

Lolium perenne: global distribution and invasion by endophytes.

Perennial ryegrass (*Lolium Perenne*) is a grass species that very well tolerates many different climatic and environmental and conditions. Therefore it is a grass species that has become very important to forage production and is nowadays widely spread and used all over the world (Hahn & McManus, 2008). In Europe, including The Netherlands, *Lolium perenne* is the most widely used forage grass(figure 1). Perennial ryegrass is one of the grasses that relies on its mutualistic relationship with endophytic fungi for its wellbeing under stressful circumstances.

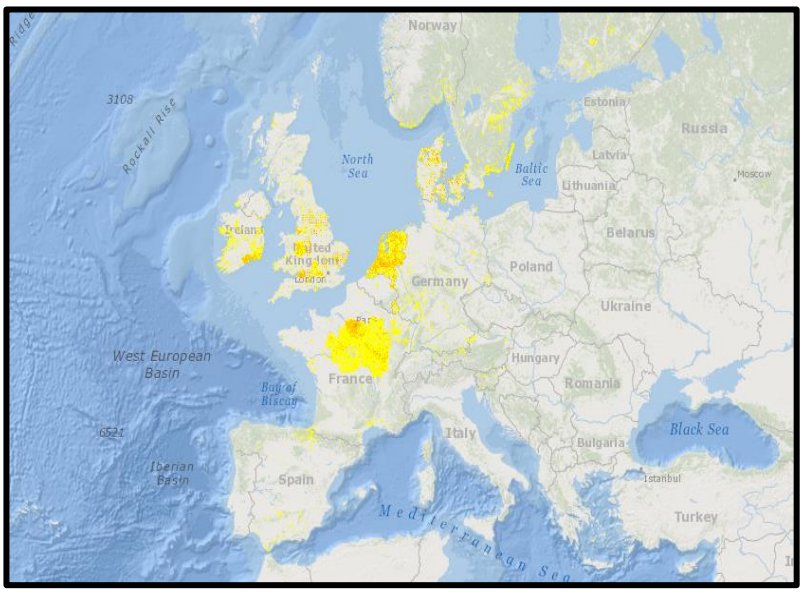

Figure 1: Distribution *Lolium perenne* in Europe 2000-2014
(Global Biodiversity Information Facility GBIF)

Lolium perenne clearly benefits of the presence of *Neotyphodium lolii*. The endophyte produces several secondary metabolites such as Peramine, paxiline and the Lolitrems that protect *Lolium perenne* against insects, bacteria, helminths and small herbivores. Peramine is the most important protector against insects, especially the Argentine stem weevil. Besides that the occurrence of *Neotyphodium lolii* in *Lolium perenne* is positively correlated with drought stress, reflecting its role in increased drought tolerance. (DiMenna et al., 2012; Gilbert et al., 2012; Rowan, 1993)

Neotyphodium lolii is most known in countries like New Zealand and Australia, however *Neotyphodium lolii* can be found in every pasture where *Lolium perenne* grows (Cheeke, 1995). Also in Europe *Neotyphodium lolii* is often found in pastures. In 1987 Latch, Porter and Tyler studied the incidence of *Neotyphodium lolli* in seeds in Europe, 53 of the 64 tested pasture-samples proved to be infected (Latch & Potter, 1987). Initially, *Neotyphodium lolii* had been allocated to the genus of Acremonium. However since *Neotyphodium lolii* was closely related to the genus *Epihcloë*, although it lacks a sexual reproduction, it was later on classified as a new genus denoted *Neotyphodium. Neotyphodium lolii* grows intercellularly in the leaf sheaths and flowering stems of the common pasture grass *Lolium perenne. Neotyphodium lolii* has an asexual life cycle. Therefore, individual plants infected with *Neotyphodium lolii* cannot infect other plants, but infected seeds will distribute the fungus. The amount of hyphae found in a plant is five times higher in the summer than in de winter, but also depending on the maturation of the plant tissue (Schardl, 2001).

Lolitrem B and its analysis in grass and hay samples

Lolitrem B, a member of the group of structurally related Lolitrems is considered as the most toxic secondary metabolites produced by *Neotyphodium lolii*. Paxilline, identified as an intermediate in the biosynthesis of Lolitrem B (Figure 2) seems to be much less toxic to farm

animals such as cattle, sheep and horses. Several lolitrems have been described, including lolitrem A, B, C, D, E and F, all of which it are indole-based alkaloids. The resemblance between the structures of paxilline and lolitrem B makes it probable that paxillin is a precursor of lolitrem B (figure 2). However lollilin might also play a role in the biosynthesis of lolitrem B (Cheeke, 1995; DiMenna et al., 2012; Munday-Finch et al., 1997; Philippe, 2016; Porter, 1995; Rowan, 1993). The structure of lolitrem B consists of a carbon skeleton with ten adjacent rings, containing one isoprenyl unit. The complex structure has several similarities with other known tremorgens from fungal origin, such as the aflatrems (fomr Aspergilli) and Penitrem A (produced by various Penicillium psiecies)

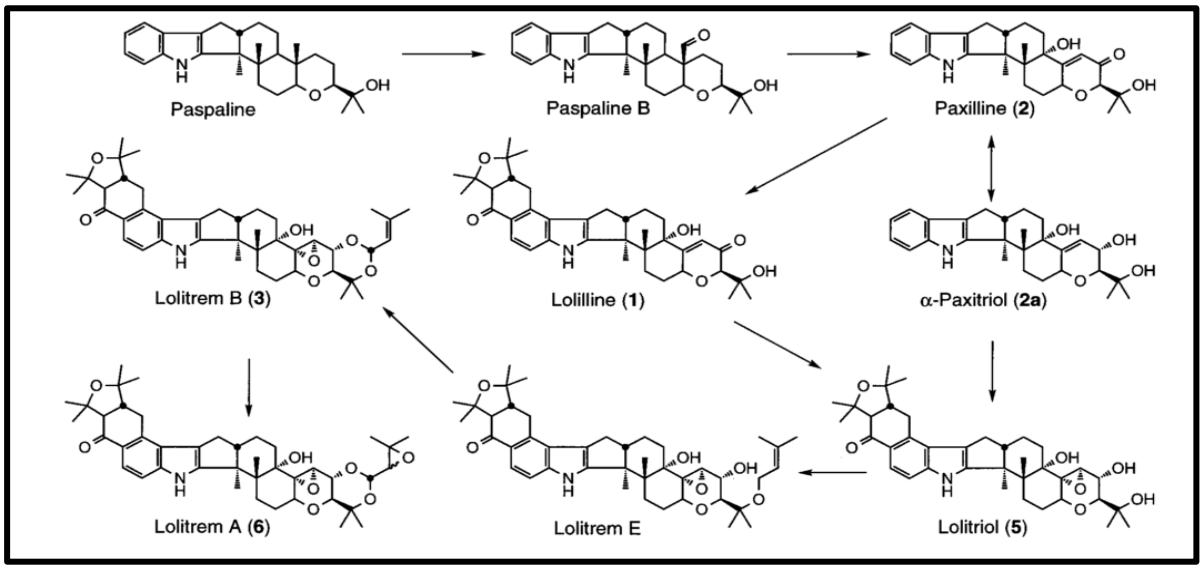

Figure 2: Structures of Lolitrem and its potential precursos in the biosynthetic pathway

The four lolitrems (A-D) as well as their precursor Paxilline exert tremorgenic effects in laboratory animal species. The most potent tremorgenic compound is lolitrem B, and the neurological disease with tremors have been observed all animal species consuming regularly larger amount of pasture grass such as sheep, cattle, horses, but also wildlife such as red deer.

The concentration of Lolitrem B is correlated to the amount of hyphae of *Neotyphodium lolii* in the grass plant and various with the season. As mentioned above, *Neotyphodium lolii* is mostly found in the leaf sheaths, flowering stems and the seeds, therefore the concentration of Lolitrem B are higher in those parts of the plant. When the *Lolium Perenne* flowers, at least 60% of the Lolitrem B concentration is found in the seeds. Another factor that influences the Lolitrem B concentration is the life cycle the plant. Higher concentrations of Lolitrem B are found in older leaves and the death leafs of the plant. So in the late summer there is a higher prevalence of Lolitrem B in grass leaves (Ball et al., 1997; DiMenna et al., 2012). Animals grazing on already overgrazed pastures are of higher risk, of Lolitrem B exposure since 75%

percent of the hyphae of *Neotyphodium lolii* are found in the lower 2 cm of the plant. Moreover, in the dry season the concentration of lolitrem B is known to increase(Hahn & McManus, 2008). The presence of has been confirmed in infected ryegrasses in Australia and New Zealand, Argentina, but also in the United Kingdom and other European countries. (Rowan, 1993, Fink-Gremmels, 2005).

The analysis of Lolitrem B in feed materials is generally conducted by chromatographic methods, and a number of different methods have been developed. Analytical procedures always start with the cleaning and extraction of the samples. The extraction step involves generally organic solvents and according to the solubility of the analyte. The cleaning of the sample after extraction by a solid phase Silica cartridge is crucial, to avoid that the extract contains numerous other molecules interfere with the identification and quantification in chromatographic methods. Separation is generally conducted by high performance liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis. The method most frequently used for the detection of lolitrem B is HPLC separation with fluorescence detection with an excitation wavelength of 268 nm and an emission of 440 nm) (Miyazaki et al., 2001; Repussard et al., 2014).

Ryegrass staggers in horses

Ryegrass staggers is the clinical description of an intoxication in grazing animals caused by the mycotoxin Lolitrem B. It is a very common disease in New Zealand and Australia, but it also is described in several other countries, including the United States and several European countries. In the Netherlands, the link between Lolitrem B and ryegrass staggers in horses was first described in 1995 (van Essen et al., 1995) involving two horses, and more attention was paid to this disease after two major outbreaks in the Netherlands in 1999 (Sloet Van Oldruitenborgh-Oosterbaan, 1999). However already in 1977 the signs of ryegrass staggers had been described, but due to the lack of methods to detect lolitrem B the causative agent could not be identified. Probably one of the first reports of ryegrass staggers is written by Gilruth in 1906. He described that in the summer and in autumn horses, as well as sheep, who grazed on ryegrass pastures, did develop signs of muscular incoordination, especially when the grass had formed seeds. The first investigation at the toxicity of *Neotyphodium lolli* was done by Neil in 1941. Chicken sheep, rats and mice were fed infected ryegrass, however without clear signs of toxicity. The next attempt was in 1958 by Cunningham, again with unconvincing result. Since then several other (myco)toxins were investigated as a possible cause for ryegrass staggers. Finally in 1981 Fletcher and Harvey proved a correlation between ryegrass staggers and the degree of endophyte infection of ryegrass. The combination of the severity of the neurological signs with the absence of severe histological lesions in the affected animals made it even more likely that ryegrass staggers is caused by a tremorgenic mycotoxin. This theory was supported by the fact that similar symptoms to those of ryegrass staggers did occur in sheep fed with known fungal tremorgens. However only in 1985 it was proven that Lolitrem B, produced by the endophyte *Neotyphodium lolli*, could cause the symptoms of ryegrass staggers (DiMenna et al., 2012; Goehring et al., 2005; Rowan, 1993).

Clinical symptoms in horses

When lolitrem B is ingested after feeding with infected forage or grazing on infected pastures the animals develop neurological signs within hours. Ryegrass staggers is seen in different animal species including cattle, sheep, deer and horses. In horses the clinical signs occur as tremors, head shaking, incoordination, staggering, swaying gait, unstable standing and fasciculations of the superficial muscles in neck and limbs. The signs can get worse when the horse is excited and forced to move, due to dysmetria and stiffen legs. In sheep this muscle tremors can progress into tetanic cramps and collapse, but these symptoms are hardly seen of the horse. When a horse collapses or even when it lay down voluntarily, it is unable to stand-up without assistants after resting. The severity of the signs can differ between horses, from poor condition and poor performance to the previously described severe clinical signs. Mildly affected horses show a head tremor, hyperesthesia and a staggering gait. Severely affected horses have more generalized tremors, and often show opisthotonus, nystagmus and salivation, and hypersensitivity to noise and light. Lolitrem B exposure has been associated with increased blood pressure and heart rate as well as with altered gastrointestinal motility (Cheeke, 1995; di Menna et al., 2012; Johnstone et al., 2012; Miyazaki et al., 2001; Philippe, 2016; Riet-Correa et al., 2013; Rowan, 1993).

Although the morbidity is high (higher than 50 %), the mortality is very low (below 10 %). Very few fatal cases have been reported mainly associated with accidents during the collapse phase. The intoxicated horses are difficult to handle and may present a risk for personnel due to the incoordination and the possibility of collapse and instability. When the horse experience severe motoric incoordination it may stagger and fall to either sides and even backwards, what makes it even more dangerous to handle these horses. When the affected horses are removed from the source of lolitrem B, the neurological signs disappear without treatment. The horse will start to recover within 2-5 days after the last intake of Lolitrem B. In same case reports, cerebellar lesions and secondary myonecrosis have been reported, but in most cases with confirmed Lolitrem B exposure and in experimental studies no persistent pathological lesions are found in the nervous system.

In horses the toxic threshold of lolitrem B has not yet been determined, but cattle do show signs after uptake about 2 ppm Lolitrem in roughages. In a Japanese report on 29 cases of ryegrass staggers in horses and cattle measured Lolitrem concentrations ranged from 0.97 to 3,7 ppb. Pronounced clinical signs of intoxication are described after an intake of 5.3 $\mu g\ g^{-1}$ (equivalent to 5.3 ppm) Lolitem B. (Cheeke, 1995; DiMenna et al., 2012;; Johnstone et al., 2012; Munday, 1985; Riet-Correa et al., 2013; Rowan, 1993).

The effect of lolitrem B has been first associated with an imbalance in the amino acid neurotransmitters GABA and Glutamate. It was assumed that Lolitrem reduces the effects of inhibitory amino acids. It was postulated that this reduction of inhibitory amino acids could lead to increased release of presynaptic neurotransmitter and prolonged depolarization, a triggering also the synaptic transmission at the motor end plate. However this hypothesis gave no satisfying explanation for the typical tremors (DiMenna et al., 2012). More recently it has

been proven that Lolitrem B acts as an inhibitor of BK-channels (calcium-activated potassium channels), which are involved in the regulation of both skeletal and smooth muscle excitation. Lolitrem B binds the α-unit of the BK-channel, thereby inhibiting K+ ion efflux from the cell. In that way it affects motor function and exerts the typical tremors. Lolitrem B binding the α-unit of the BK-channels is concentration-dependent (Dalziel et al., 2005; Imlach et al., 2008; Johnstone & Mayhew, 2013; Philippe, 2016).

Diagnostic options in cases of ryegrass staggers

The diagnosis of ryegrass staggers is based on the typical clinical signs. The neurological signs are relative specific, and form a clear indication for a further diagnostic work-up. The neurological signs can be scored (table 2). Another indication for ryegrass staggers is the appearance of clinical signs in several horses in the same stable and the fact that a new batch of hay has been used recently. Clinical investigation will show an unaffected consciousness of the affected horse and the absence of fever, and the typical staggering gait during clinical examination. These findings can lead to a probable diagnosis and should always accompanied with the immediate withdrawal and replacement of the hay as the major source of Lolitrem B exposure. The affected horses will show improvement within 2 days . For confirmation of the clinical diagnosis, a hay sample should be analysed for the presence of Lolitrem B. More recently, also serum analyses of Lolitrem B have been described. However, as yet there is no validated test for lolitrem B in blood or other body fluid. Johnstone et al. used an ELISA test for both plasma and urine to measurements for the confirmation of lolitrem B exposure. The plasma level was proven to be increased, however the measured values did not correlate with the severity of the clinical signs. In urine only traces of lolitrem B where found. In the absence of complete kinetic data on lolitrem B in horses, in clinical practice the analysis of feed samples (hay) is still the most common method to confirm the clinical diagnosis of ray-grass intoxications. (Goehring et al., 2005; Johnstone et al., 2012). Feed analysis also support the advice to be given to the horse owner, as increasing the storage time will reduce Lolitrem B values, but also this depends on the initial concentrations measured.

Table 2 Scoring system ryegrass staggers (Johnstone et al., 2012)

SCORE	NEUROLOGICAL SYNDROME
0	No clinical signs
1	Slight sway and/or slight tremor when feeding and after exercise; no ataxia without blindfolded.
2	Obvious sway and/or tremor when feeding and after exercise; mild to moderate disequilibrium when blindfolded.
3	Obvious sway and/or tremor when feeding and after exercise; mild to moderate disequilibrium and postural deficits when blindfolded; mild ataxia when not blindfolded.
4	Sever tremors and ataxia.
5	Collapse and cerebellar fits.

Therapeutic intervention and preventive measures

The symptoms caused by lolitrem are reversible and a causal treatment is not essential under clinical conditions. Horses will recover spontaneously already within 2 days when the intake of Lolitrem B is discontinued, but it may take 2 weeks or more, until the horse is fully recovered. Even for severe cases, a proper treatment is currently not available. Imlach et al. suggested that BK channel activators may be an option since the symptoms are caused by inhibition of these channels (Imlach et al., 2008) but the broad distribution of BK-Channels in the body bears the risk of a diversity of side effects, and hence their application seem not to be justified (Bentzen et al., 2014) .

The main objective after a clinical diagnosis is the identification of the source of Lolitrem. While is general, in Europe Lolitrem appears generally only in hay at clinical relevant concentrations, in some cases overgrazed grassland in late summer may be also the cause. As the presence of protective endoptyte metabolites in grassland is less important in Europe (as compared to Australia and new Zealand) , managerial measures to reduce the risk for horses include:

- Avoidance of hay from grass seed operations, as grasses for ornamental purposes (including also golf course and sport fields) are generally endophyte positive.
- Avoidance of overgrazing of natural rye-grass pastures (on sandy and dry soils) in late summer
- Avoidance of feeding very fresh (newly harvested) hay, as storage time will reduce the levels of Lolitrem B due to the normal microbiological activity in hay. This is in contrast to almost all other mycotoxins, which increase in concentrations during storage.
- Haylage or silage has a higher microbiological activity, and hence allows a safer use of harvested rye grass.

This degradation during storage is accelerated by higher temperatures (>20ºC) and higher humidity (>70%). In contrast, when infected forage is stored cool (<5ºC) and dry (humidity < 11%) the toxin may persist in dangerous concentrations up to 15 years. Since it is hard to predict the rate of degradation of Lolitrem B under field conditions, in cases of noted intoxications, it is recommended to store the hay for at least 3 months and re-test it prior to use to ensure that Lolitrem B concentrations have been declined to safe levels (Goehring et al., 2005; J. Fink-Gremmels 2005; Nollet & Vanschandevijl, 2007).

There have also been several studies to breed a specie of *Lolium perenne* that is not infected with *Neotyphodium lolli*. While this is in principle possible, the lack of protection (by endophyte metabolites) against several insects and stressful conditions of the new varieties restricted there use in practice (DiMenna et al., 2012).

Investigations in The Netherlands
Materials and Methods

Sampling:

20 hay samples were taken at random from 20 different stables in the period from 5 Septembe till 15 October 2014 and tested with the HPLC method according to Gallagher, Hawkes, & Stewart, 1985, with some adaptations(Gallagher et al., 1985; Repussard et al., 2014; Turner et al., 2009).

Sample extraction:

The hay (taken at random from a the batch of hay present at the farm) was cut and grinded and then sieved. Only the mash smaller than 0.473 mm (Mash size as given) where used. From each sample 0.5 gram was weighed and mixed with 7.5 ml MQ water in 15 ml glass tubes and soaked for 1 hour. Then the suspension were placed in an ultrasonic bath for 5 minutes. For extraction, 5 ml chloroform and 2.5 ml methanol was added and the sample mixed on a rotator for 1 hour. After sedimentation, the supernatant was collected, and centrifuged again for 10 minutes at a temperature of 4°C. After centrifugation the three different fluid layers can be distinguished and an aliquot of 1 ml was taken from the organic phase at the bottom and evaporated to dryness under a stream of nitrogen.

Sample clean-up: :

The Bakerbond silica column was rinsed with 3 ml dichloromethane. The dried extract was dissolved in 0.5 ml dichloromethane and passed through the column, and the column rinsed again with 0.8 dichloromethane. The combined effluents were dried again under a stream of nitrogen and finally dissolved in 250 µl mobile phase (Dichloromethane/acetonitrile).

HPLC analysis:

For HPLC separation a Luna silica 5um 250 * 4.6mm column was used. The mobile phase consisted of Dichloromethane/acetonitrile (75:25) set at a flow rate of 1.2 ml/min. For the detection a JASCO fluorescence detector was used set at a excitation wavelength of 268 and a emission wave length of 440. Peak areas were recorded and compared with a pre-established standard curve. Spiked samples were used to confirm peak identification where appropriate.

Standard curve Lolitrem B:

To create a standard curve a stock solution of 1 ng/µl was prepared and serially dilated to a final concentration of 0.04 ng/µl An example of a standard curve is given below (Table 3)

Table 3: *Standard curve for Lolitrem under the experimental conditions described above.*

Concentration ng/ul	Area under the peak
0.04	4.39
0.08	10.5
0.16	20.54
0.32	51.45
0.64	100.49
0.8	132.13
1	166.84

The plot (Figure 3) presents the standard cure and regression line representing the formula: y = 169.19x – 4.0001. The regression line (corrected for the dilution factor) was used to calculate the concentrations of Lolitrem in the samples.

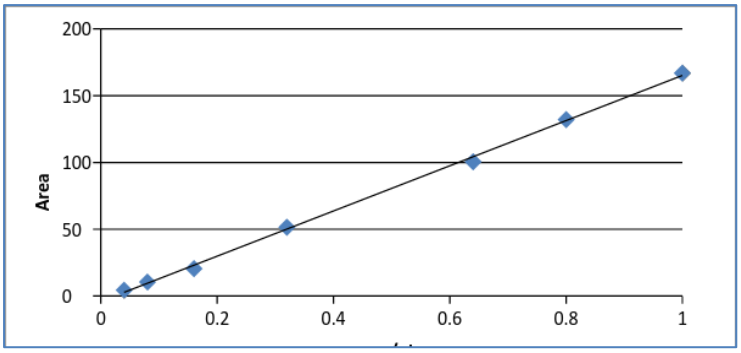

Figure 3: Plot of the standard curve

Results of at random collected hay samples

The results of at random collected hay samples from different locations in the Netherlands are presented in Table 4.

Table 4: *HPLC results of at random samples*

Sample	area under the peak	Concentration (ng/ul) = ppm
1 (Hay)	0.27	0.00631
2 (Silage)	0.71	0.00696
3 (Silage)	0.15	0.00613
4 (Silage)	0	0
5 (Hay)	0	0
6 (Silage)	0	0
7 (Silage)	0.18	0.00618
8 (Silage)	0.16	0.00615
9 (Hay)	0.45	0.00658

10 (Hay)	0	0
11 (Silage)	0	0
12 (Hay)	0	0
13 (Hay)	0	0
14 (Silage)	0.09	0.00604
15 (Hay)	0.04	0.00597
16 (Hay)	6.50	0.01551
17 (Hay)	0.11	0.00607
18 (Hay)	0.20	0.00621
19 (Silage)	0,02	0.00594
20 (Hay)	0.04	0.00597

For comparison, the results of historical samples, analysed upon request of a veterinarian/horse owner were summarized in the following table (table 5). This table present the range of concentrations found. In total 50% of the sample were found to exceed the common threshold values for clinical intoxication of 1.2 ppm, whereas all concentrations above 0.8 ppm were also reported, as these samples have to be considered as suspected.

Table 5: *Summary of historical samples analyses at the veterinary faculty (only values above the clinical acute reference concentration of 0.8ppm are reported)*

Location	Concentration (mg/kg) = ppm
Bergen op Zoom	1.7
Numansdorp	2.4
Ridderkerk	1.5
Lelystad	1.7
Steenbergen	1.4
Bergen op Zoom	1.5
Breda	3.1
St Oedenrode	2
St Oedenrode	2
Lekkerkerk	1.5
Heeswijk	6.6
Breda	3

Meppel	1.2
Nederland	3.5
St Oedenrode	2.1

Data show no clinical relevant Lolitrem concentration in the at random samples (table 4). Previous analyses, conducted upon request on suspicious hay samples showed a concentrations varying between 1.2 - 6.6. ppm. The results in table 5 represent only the data considered of clinical relevance. Early investigations had indicated the threshold of toxicological concern for horses consuming Lolitrem contaminated hay in The Netherlands is expected to be between 0.8 and 1.2 ppm (Fink-Gremmels and Bull, 2000). For this calculation a normal hay consumption was assumed, which varies, however, in practice, resulting in the range given. Next to these values, in the same period 14 other sakples were tested for the presence of Lolitrem upon request of horse owners, but in all these samples, the Lolitrem B concentrations measured remained well below 0.8 ppm. Whether or not the horses displayed the typical signs of ryegrass staggers of any other neurological conditions is not known.

Discussion

The aim of the study was to gain a better insight of the risk of Lolitrem B exposure and adverse effects in horses with particular emphasis on the situation in the Netherlands. In order to analyse this risk, the literature was reviewed and data from measurements from 2004 to 2014 on hay samples in the Netherlands are analysed together with historical samples from horse farms with clinical signs of lolitrem B intoxication.

Prevalence and threshold of concern of lolitrem B exposure of horses in the Netherlands

The positive historical samples from horse farms with clinical signs show a range of 1.2 to 6.6 ng/ul. Previous investigations in the Netherlands suggested that the threshold of concern seems to range between 0.8 – 1.2 ppm in hay (Fink-Gremmels and Bull, 2000). The concentration lolitrem B in the at random taken samples varied from 0 to 0.01551 ppm and therefore all samples tested negative for lolitrem B. As also about 50% of the historical samples tested negative, these findings indicate a low prevalence of lolitrem B in hay in the Netherlands. Only few outbreaks of ryegrass staggers in the Netherlands have been reported in the literature, which is also indicative for a low prevalence. However, It should be noted that some outbreaks of ryegrass staggers in the Netherlands may not have been followed by analysis on lolitrem B, since history and recovery of the affected horses after removal of the infected hay resulted in spontaneous recovery in the horses, and hence the owners were not interested in further (diagnostic) investigations.

Clinical implications and differential diagnoses

Clinicians in the Netherlands should however consider ryegrass staggers as an important

differential diagnosis in cases of neurological signs associated with tremor and staggering in horses. Lolitrem staggers require no further treatment after identification and removal of the contaminated hay and have an excellent prognosis for full recovery. In contrast, most of the other clinical condition require intensive treatment and have generally a very poor prognosis. As these differences are crucial for the veterinarian, table 6 presents an overview of neurological disorders that need to be considered. The differential diagnoses should be based on the clinical signs and disease epidemiology. Infectious diseases should be rapidly diagnosed to prevent further spreading of the disease among the population of horses. Some of the mentioned disease have serious implication for the horse owners (such as notifiable disease such as EHV-Infections) or have a very poor prognosis. Hence in any of these cases, Lolitrem intoxications should be rules out before further actions are taken.

Table 6: *Differential diagnosis of Ryegrass staggers, classified by diet-related, anatomical location, and infectious nature (based on (Alleman, 2011; Barr et al., 2001; Reed et al., 2010; Riet-Correa et al., 2013; Rech and Barros, 2015)*

Disease/ agent:	congruent symptoms to Ryegrass staggers:	Incongruent symptoms to Ryegrass staggers:	Exclusion by:
Diet related			
Leukoencephalomalacia (Fumonisin b)	incoordination, lethargy	aimless walking, intermittent anorexia, depression, blindness, and head pressing,	Necropsy lesions: unsymmetrical malacia of the white matter and determination of fumonisin in the feed
Bermuda grass staggers (Paspalitrems)	tremors, head shaking, incoordination, staggering, swaying gait, unstable standing and muscle fasciculations		Not occurring in The Netherlands
Paspalam grass staggers (Paspalitrems)	tremors, head shaking, incoordination, staggering, swaying gait, unstable standing and muscle fasciculations		Not occurring in The Netherlands
Nigropallidal encephalomalacia	lack of coordination	impairment of eating and drinking, mastication, and deglutition	No reported occurrence in The Netherlands/ typical necropsy lesions
Hypocalcaemia	muscle fasciculations, high-stepping gait,	ileus, colic-like signs due to muscle pain, sweating,	Plasma ionized calcium

	tremors, staggering, ataxia, and recumbency	salivation, diaphragmatic flutter, “tetany”, seizures, and trismus.	measurement of blood sample
Equine motor neuron disease	trembling, muscle fasciculations	base-narrow standing, excessive recumbency, muscle atrophy, and weight loss	Biopsy of the sacrocaudalisdorsalis medialis muscle
Equine grass sickness	fine muscular fasciculations, altered gastro-intestinal motility	generalised or patchy sweating, pyrexia, severe dysphagia, and bilateral ptosis	full-thickness ileal biopsies obtained at exploratory celiotomy
Diffuse CNS: infectious			
EHV-1	Spinal ataxia	cranial nerve abnormalities, involvement cauda equina	Cerebrospinal fluid shows signs of infection/ PCR on EHV-1/ serology
Equine protozoal myelitis (*Sarcocystis neurona*)	progressive ataxia	cranial nerve deficits	Not occurring in the Netherlands / necrotic lesions in the CNS
Verminous encephalomyelitis	ataxia, recumbency	head tilt, circling, blindness, hyperesthesia, stiff neck, head pressing, seizures, and coma	Histopathology of the brain and spinal cord
Diffuse CNS: non-infectious			
Thiaminase	weight loss, incoordination, ataxia, wide-based stance, muscle fasciculations, tremors	death	Clinical signs/ diet and medical history
Locoism	trembling, ataxia, hyperesthesia	depression, and behavioural unpredictability	Not occurring in The Netherlands
Cerebellar: non-infectious			
Cerebellar abiotrophy	intention tremors of the head, ataxia, dysmetria	age of occurrence: foals < one years of age race: Arabians	Clinical symptoms, age and race
Chronic methyl mercurial poisoning	incoordination, dysmetria, head nodding, lethargy	anorexia, exudative dermatitis, and laminitis	
Spinal: non-infectious			
Cervical vertebral malformation	symmetrical ataxia and dysmetria	upper motor neuron paresis	X-rays/ neurological exam (specific site in

			spinal cord)/ compression lesions in spinal cord
Equine degenerative myeloencephalopathy	ataxia	weakness and spasticity, occurring in young horses < 2 years	Histopathologic lesions/ age

Conclusions

Lolitrem B intoxications are an important differential diagnoses when horses are presented with neurological signs including tremors and incoordination. The diagnosis can be easily confirmed by analysis of feed samples and recently also serum sample measurements has been discussed as rapid method. In contrast to other neurological diseases, Affected animals require no specific pharmaco-therapeutic treatment other that an immediate withdrawal of the contaminated hay (ore pasture). As many horses showed a higher sensitivity to light and noise, it is recommended to keep horses in-house during the first days. Horse owners should be informed that the symptoms will disappear within 2 to 3 days, but horses should not be used for sports (jumping) for a period of about 3-4 weeks, as incoordination may persist sub-clinically for a longer period.

Contaminated hay needs not to be entirely discharged, as during longer storage (> 3 months) a spontaneous microbiological degradation will reduce the Lolitrem B concentrations. As this degradation and inactivation depends on storage temperature and microbiological activity, it is still recommended to analyse the hay again prior to use, when initial concentrations were high.

For the veterinary practitioner it remains important to consider Lolitrem B intoxications as a differential diagnosis whenever horses are presented with neurological signs, including tremors.

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